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ORIGINAL ARTICLE

The oxidative stress status in diabetes mellitus and diabetic nephropathy

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Abstract The aim of this study is to assess the oxidative stress status in diabetes mellitus (DM) and diabetic nephropathy. The study group comprised 40 control subjects, 40 type 2 DM patients without complications and 37 diabetic nephropathies. Compared with control subjects, superoxide dismutase, glutathione peroxidase, catalase, vitamin C were decreased ($P < 0.01$). There was a significant increase in serum malondialdehyde (MDA), conjugated diene (CD), advanced oxidation protein products (AOPP), protein carbonyl (PC) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) in diabetes patients when compared with normal subjects ($P < 0.01$). Moreover, these indexes were much higher in diabetic nephropathy than that of diabetic patients without vascular complications ($P < 0.05$, $P < 0.01$). There was a significant correlation between the serum glucose levels and PC, 8-OHdG ($P < 0.05$, $P < 0.01$). There were highly significant positive correlation of CD and MDA, AOPP and PC ($P < 0.01$). Plasma AOPP levels had a significant correlation with PC levels ($P < 0.01$). Our findings suggested that diabetes patients have more severe oxidative stress than normal persons and higher oxidative stress in diabetic nephropathy than those in patients without complications. Oxidative stress may play an important intermediary role in the pathogenesis of diabetes complications.

Keywords Diabetes mellitus · Diabetic nephropathy · Oxidative stress

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Introduction

Diabetes mellitus (DM) is a chronic metabolic syndrome which has reached epidemic proportions worldwide and represents a serious public health concern. Type 2 diabetes is characterized by defective insulin secretion in pancreatic β-cells in response to glucose and by deficiencies in the action of insulin on its target tissues. Vascular complications are the main leading cause of morbidity and mortality in DM. The prevalence of DM, particularly type 2 DM, has rapidly increased in industrialized and many developing countries. It is estimated that it will affect approximately 366 million people by 2030. In China, it is increased very rapidly in the recent years. Currently, type 2 diabetes affects up to 20.8 million people, and this figure is expected to approach 42.3 million by 2030 in China [1].

Enhanced oxidative stress contributes to the pathogenesis of DM. Type 2 diabetes is associated with increased oxidative stress, which may contribute to microvascular and macrovascular complications [2]. Under normal physiological conditions, there is a balance in the generation of oxygen free radicals and antioxidant defense systems used by organisms to deactivate and protect themselves against free radical toxicity. Impairment in the oxidant/antioxidant equilibrium creates a condition known as oxidative stress. In general, oxidative stress, including reactive oxygen species (ROS), can damage cellular macromolecules, leading to DNA and protein modification and

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lipid peroxidation. It causes tissue degeneration, particularly in vascular system. Mechanisms involved in the increased oxidative stress in diabetes include not only increased oxidative damage products of protein, lipid and DNA, but also changes in the tissue content and activity of antioxidant defense systems. Increasing evidence in both experimental and clinical studies suggests that there is a close link between hyperglycemia, oxidative stress and diabetic complications. It has been suggested that oxidative stress plays a key role in the pathogenesis of vascular complications, both microvascular and macrovascular [3].

Diabetes-associated nephropathy is one of the major chronic complications of type 2 diabetes and finally progresses to end-stage renal disease requiring dialysis therapy. The pathogenesis of diabetic nephropathy is multifactorial, and the precise mechanisms are unclear. A number of studies in vitro and in vivo suggest that oxidative stress is increased in diabetic nephropathy patients and animal models of diabetes and oxidative stress may contribute to the pathogenesis of diabetic nephropathy [4–6]. There remains controversy, however, as to whether there is an early link between hyperglycemia and renal disease or develops as a consequence of other primary pathogenic mechanisms. The detailed mechanism remained to be elucidated.

Taking these studies into account, our study aims to evaluate the oxidative stress status in type 2 diabetes mellitus and diabetic nephropathy patients and investigate the relationship between oxidative stress and diabetes and its complication.

Subjects and methods

The studied group consisted of 67 patients with type 2 diabetes mellitus, who were recruited from the First Affiliated Hospital, Harbin Medical University. The diabetic patients included 40 diabetes mellitus patients without diabetic complications, such as nephropathy, neuropathy, hypertension, cardiac ischemic disease, and peripheral vasculopathy. Thirty-seven patients with diabetic nephropathy (DN), defined as a persistent albumin excretion rate >200 μg/min in sterile urine, concomitant retinopathy, and no other renal disease or heart failure. All patients were diagnosed according to the World Health Organization diagnostic criteria for type 2 diabetes. All patients were on a diabetes diet and were treated with insulin. We excluded the patients who had acute and chronic infections, fever, malignancy, acute and chronic nephritis, cirrhosis and congestive heart failure. Forty healthy control subjects were recruited from the health examination center of the First Affiliated Hospital, Harbin Medical University. None of the control subjects was taking any medication. The three groups of subjects were matched for age and sex. The study protocol was approved by the Ethics Committee of the Harbin Medical University. All subjects gave written informed consent to participate in this study. The characteristics of the study population are shown in Table 1.

Blood sampling

Venous blood samples were collected after 12 h overnight fasting from each subject. The samples were placed on ice and centrifuged within an hour at 3,500 rpm, 4°C for 15 min, and the supernatants were stored at −20°C and determination of the samples occurred within 3 months.

Measurement of antioxidants

We measured superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) activity in serum

Table 1 Clinical characteristics of diabetes mellitus patients and health control

	Diabetes mellitus	Diabetic nephropathy	Health group
n	40	37	40
Age (years)	50.78 ± 12.21	52.81 ± 11.50	52.38 ± 11.12
Sex (F/M)	23/17	21/16	22/18
HbA_{1c} (%)	8.07 ± 1.61	8.47 ± 1.86	–
Insulin (mmol/l)	11.84 ± 6.66	10.86 ± 5.69	–
Serum glucose (mmol/l)	10.93 ± 2.38[a]	11.48 ± 3.00[a]	4.93 ± 0.41
Cholesterol (mmol/l)	4.97 ± 0.97	5.47 ± 1.42	5.07 ± 0.70
Triglycerides (mmol/l)	2.27 ± 1.40[a]	2.58 ± 1.43[a]	1.08 ± 0.39
Duration of disease (months)	32.18 ± 35.77[b]	68.43 ± 58.88	–
Systolic blood pressure (mmHg)	130.61 ± 5.83	135.18 ± 8.31	129.56 ± 7.28
Diastolic blood pressure (mmHg)	81.42 ± 4.08	84.62 ± 8.88	81.49 ± 5.17
Serum creatinine (μmol/l)	67.13 ± 10.94[c]	72.58 ± 24.77[a]	59.25 ± 12.12
C-peptide (ng/ml)	1.84 ± 0.92	2.02 ± 1.23	–
Urinary albumin excretion rate (μg/min)	<20	>200	<20

Data are mean values ± SD

[a] $P < 0.01$ versus health group

[b] $P < 0.01$ versus diabetic nephropathy

[c] $P < 0.05$ versus health group

according to the methods of spectrophotometry. Concentrations of vitamin C and E were determined by reverse-phase high-performance liquid chromatography.

Measurement of lipid peroxidation products

Quantitative examination of products of lipid peroxidation included assays for conjugated dienes (CD) and malondialdehyde (MDA). CD was determined according to the method of Ward et al. [7]. MDA levels were measured spetrophotometrically by using thiobarbituric acid-reacting substance (TBARS) production.

Measurement of protein damage products

Products of protein oxidation included advanced oxidation protein products (AOPP) and protein carbonyl content. AOPP were quantified as described by Witko-Sarsat [8]. Protein carbonyl (PC) concentrations in serum were measured by the spectrophotometric assay.

Measurement of DNA damage product

DNA damage was evaluated by measuring the 8-hydroxydeoxyguanosin (8-OHdG) content in serum. Serum 8-OHdG was measured by using enzyme-linked immunosorbent assay method.

Other biochemical parameters

Blood glucose, triglycerides and cholesterol were determined using routine clinical chemical assays.

Statistical analysis

Data are presented as mean ± SD. All experimental data in this study were statistically analyzed with SAS 9.13. The statistical significance was evaluated using an unpaired Student's t test. Results were considered significant at $P < 0.05$.

Results

As shown in Table 1, the study groups were well matched for age, sex. The diabetes mellitus patients had significantly elevated levels of fasting blood glucose and triglycerides. And the diabetes duration of diabetic nephropathy was longer than that of diabetes mellitus without complications. There was a significant increase in serum creatinine in type 2 diabetes mellitus patients with or without microvascular complications when compared with normal subjects.

As shown in Table 2, the levels of antioxidative enzymes in the serum of diabetes patients were decreased. Compared to the control group, the activities of SOD, GSH-Px and CAT in diabetes mellitus patients were lower, respectively; however, no significant difference was found between diabetes mellitus patients without microvascular complications and diabetic nephropathy. Serum vitamin C concentrations were also significantly lower in diabetic patients compared to the control group, and there was no significant differences between diabetics mellitus patients without microvascular complications and diabetic nephropathy. The serum vitamin E concentrations were reduced in diabetes patients, but the differences with those of the healthy controls were not statistically significant.

Oxidative stress parameters including MDA, CD, AOPP, PC, and 8-OHdG are listed in Table 3, showing that the products of oxidative stress in diabetes patients were significantly altered compared to the controls. There was significant increase in serum MDA and CD in diabetes patients when compared with normal subjects ($P < 0.01$). Also, diabetic nephropathy patients have significantly higher levels of MDA and CD compared to those without vascular complications ($P < 0.01$). Serum AOPP and PC in diabetes patients have significantly higher than that of the controls ($P < 0.01$). Levels of serum AOPP and PC were further higher in patients with nephropathy compared to those without vascular complications ($P < 0.01$). There were significant increase in serum 8-OHdG in type 2 diabetics mellitus patients with or without microvascular complications compared with normal subjects ($P < 0.01$).

Table 2 The results of antioxidative enzymes and antioxidants

	Health group	Diabetes mellitus	Diabetic nephropathy	P value
SOD (U/ml)	125.66 ± 30.32	104.72 ± 18.35[a]	99.82 ± 16.41[a]	<0.001
GSH-Px (U/ml)	153.93 ± 24.66	131.02 ± 33.74[a]	133.05 ± 26.19[a]	0.001
CAT (U/ml)	6.60 ± 1.50	5.21 ± 1.46[a]	5.31 ± 1.49[a]	<0.001
Vit C (mg/l)	86.23 ± 20.60	59.33 ± 18.55[a]	58.00 ± 15.25[a]	<0.001
Vit E (mg/l)	18.67 ± 5.61	16.63 ± 4.05	16.85 ± 4.02	0.104

Data are mean values ± SD

[a] $P < 0.01$ versus health group

Table 3 Products of oxidative stress in serum of diabetes patients and control

	Health group	Diabetes mellitus	Diabetic nephropathy	*P* value
MDA (nmol/ml)	3.16 ± 1.21	6.30 ± 2.32[ab]	7.30 ± 2.60[a]	0.040
CD (OD)	0.304 ± 0.107	0.445 ± 0.214[ab]	0.558 ± 0.252[a]	0.015
AOPP (μmol/l)	15.90 ± 5.35	24.52 ± 8.47[ab]	28.25 ± 7.73[a]	0.027
PC (nmol/ml)	3.07 ± 0.93	3.93 ± 1.42[ab]	4.62 ± 1.15[a]	0.013
8-OHdG (ng/ml)	5.89 ± 1.68	23.40 ± 5.31[ac]	26.97 ± 6.46[a]	0.005

Data are mean values ± SD

[a] $P < 0.01$ versus health group

[b] $P < 0.05$ versus diabetic nephropathy

[c] $P < 0.01$ versus diabetic nephropathy

Moreover, diabetic nephropathy patients had significantly higher 8-OHdG compared with diabetic patients without vascular complications ($P < 0.05$).

Correlation analysis revealed significant and positive correlation between serum glucose levels and PC ($R = 0.305$, $P = 0.007$), 8-OHdG ($R = 0.255$, $P = 0.047$). Duration of diabetes significant positively correlated with MDA, AOPP and 8-OHdG ($R = 0.225$, $P = 0.050$; $R = 0.239$, $P = 0.036$; $R = 0.309$, $P = 0.011$, respectively). Duration of diabetes significantly negatively correlated with SOD ($R = -0.197$, $P = 0.006$). Significant and positive correlation was found between CD and MDA, AOPP and PC($R = 0.454$, $P < 0.001$; $R = 0.354$, $P = 0.002$; $R = 0.251$, $P = 0.028$, respectively). A positive correlation was also obtained between AOPP and PC ($R = 0.364$, $P = 0.001$). And there was positive correlation between 8-OHdG and MDA, CD and AOPP($R = 0.482$, $P < 0.001$; $R = 0.348$, $P < 0.001$; $R = 0.452$, $P < 0.001$, respectively).

Discussion

In this study, we investigated the oxidative stress status in type 2 diabetes mellitus and its complication, and whether any difference exists between diabetes mellitus with and without nephropathy. The present study shows that there is severe oxidative stress in diabetes mellitus, especially in patients with diabetic nephropathy.

Hyperglycemia plays a critical role in the development and progression of diabetic nephropathy, but the mechanism by which hyperglycemia results in the development of diabetic nephropathy is not clear. The possible sources of increased oxidative stress might include increased generation of free radicals or impaired antioxidant defense system. Enhanced levels of free radicals found in diabetes [9, 10]. Enzymatic and non-enzymatic antioxidant defense systems include SOD, CAT, GSH-Px, vitamin E, vitamin C and vitamin A and β-carotene. They are localized in varying locations in cells and play important roles in scavenging oxygen free radicals. These antioxidants play important roles in the normal metabolism and health states in the human's bodies and demonstrated to be altered in diabetes [11–14].

Decreasing of antioxidants may break the balance between pro- and anti-oxidant and cause cellular damages and ultimately malignant transformation. Many studies have examined the changes in both extra and intracellular antioxidants and oxidant status in diabetes patients [11–14]. In our study, we have found that there was a significant decrease in the activity of SOD, GSH-Px and CAT in serum of diabetes patients. And the non-enzymatic antioxidants, such as vitamin C were significantly reduced in diabetes patients. But these results were not significantly different between diabetes mellitus patients without microvascular complications and diabetic nephropathy. These results show that there was oxidative stress in diabetes patients and support the hypothesis of radical-mediated injury in diabetes.

The present study shows that the biomarkers of oxidative stress were increased in diabetes and its complications [6, 13–16]. In general, oxidative stress, including ROS, can cause oxidative damage to DNA, proteins, and lipids, leading to DNA and protein modification and lipid peroxidation, and many clinical conditions are associated with increased indices of oxidant stress.

Lipid peroxidation is initiated by free radicals attack of membrane lipids, generating large amounts of reactive products, which have been implicated in diabetes and its complications. MDA and conjugated dienes are the two markers used widely. Conjugated dienes is the initial formation of a lipid peroxide. MDA is a decomposition product of peroxidized polyunsaturated fatty acids. Increased serum MDA and conjugated dienes levels have been found in diabetes mellitus patients [11–14]. In our study, we found that diabetes mellitus patients had significantly higher MDA and conjugated dienes when compared

with control subjects. Therefore, the concentration of MDA and conjugated dienes in patients with nephropathy was significantly elevated in comparison to diabetic patients without nephropathy.

The oxidation of protein plays an essential role in the pathogenesis of a large number of degenerative diseases, which is now widely recognized. Biomarkers of protein oxidation are often applied when a battery of markers of oxidative stress status is being studied [16–20]. Many different types of protein oxidative modifications can be induced by free radicals. However, protein carbonyl content is the most general and well-used biomarker of severe oxidative protein damage. Also, protein oxidation is a useful marker to evaluate oxidative stress in vivo. Carbonyl group formation is considered an early and stable marker for protein oxidation. The concentration of protein carbonyl was stable, yielded quantitative results, and appeared to reflect disease endpoints in a biologically significant way. Moreover, increased protein carbonyl has been found in diabetes patients [14, 18].

Recently, a new marker of protein oxidation, advanced oxidation protein products (AOPP), has begun to call the attention of some investigators. AOPP were described by Witko-Sarsat et al. [8] for the first time. They are formed during oxidative stress by the action of chlorinated oxidants, mainly hypochlorous acid and chloramines (produced by myeloperoxidase in activated neutrophils). AOPP are elevated in patients with diabetes where they correlated with markers of oxidative stress [14–16, 18]. In our study, we have also determined the level of AOPP and protein carbonyl. We can conclude that both protein carbonyl and AOPP are elevated in patients with diabetes mellitus with marked differences between patients with complications or without complications. The increase of both parameters is more pronounced in patients with diabetic nephropathy.

Numerous studies have shown that oxidative DNA damage links pathogenically to a variety of aging-associated degenerative diseases such as cancer, coronary heart disease and diabetes [21]. ROS can cause strand breaks in DNA and base modifications, including the oxidation of guanine residues to 8-hydroxydeoxyguanosine (8-OHdG), an oxidized nucleoside of DNA, is the most frequently detected and studied DNA lesion. It has been regarded as a novel biomarker of oxidative DNA damage in vivo. Hyperglycemia itself, by contributing to increased generation of ROS and oxidative stress, would lead to oxidative damage. In nuclear and mitochondrial DNA, 8-OHdG is released into blood and urine after excised from DNA by the repair enzyme. Previous studies had shown that diabetic patients had higher levels of 8-OHdG [22, 23]. Moreover, the content of 8-OHdG in the urine and mononuclear cells of type 2 diabetic patients with either retinopathy or nephropathy was much higher than those in patients without complications. Urinary 8-OHdG excretion was significantly correlated with severity of tubulointerstitial lesions in diabetic nephropathy and it had been reported as a sensitive biomarker of oxidative DNA damage [6, 24]. In our research, we observed similar association between serum 8-OHdG and diabetic nephropathy. Therefore, it could be concluded that serum 8-OHdG could be act as a sensitive biomarker for the diabetic nephropathy, too.

We have found some positive correlations between serum glucose levels and protein carbonyl and 8-OHdG. Hyperglycemia itself contributes to increased generation of ROS and increased oxidative stress would lead to oxidative damage. Nishikawa et al. [25] showed high blood glucose levels in vitro impair cellular DNA repair and increase DNA cleavage and blockade of hyperglycemia-induced ROS production would reverse the pathways implicated in diabetic angiopathy in cultured endothelial cells. We have also found some positive correlations between lipid peroxidation, protein damage and DNA oxidations suggesting that either parallel or interactive macromolecular oxidative damage may occur in the generation and development of diabetes mellitus and its complication.

In conclusion, we found that there was severe oxidative stress in the diabetes than in normal persons. We believed that the patients with diabetes had decreased antioxidative system and increased products of oxidative damage versus control subjects. Moreover, the content of oxidative damage of DNA, protein and lipid in the serum of type 2 diabetic patients with nephropathy were much higher than those in patients without complications, suggesting higher oxidative stress in patients with diabetic nephropathy. Therefore, the measurement of products of oxidative stress rather than antioxidative enzymes seems to be the best biomarkers for diabetic complication and assessment of oxidative stress in diabetic patients may be important for the prediction and prevention of diabetic complications.

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Conflict of interest statement None to declare.

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